Translated from Japanese

(19) Japanese Patent Office (JP)

(12) Official Gazette for Kokai Patent Applications (A)

(11) Patent No. H1-254699

(43) Disclosure Date: October 11, 1989

(51)	Int. Cl.[4]		Identification Symbol	JPO File No.
	C 07 K	7/40		8318-4H
	A 61 K	37/26	ADP	8615-4C
//	C 07 K	99:26		

Request for examination: Not yet submitted

Number of Claimed Inventions: 5 (5 pages total)

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(54) Title of the Invention: Insulin derivative and applications for use

(21) Application No. S63-83912

(22) Application Date: April 5, 1988

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SPECIFICATION

1. Title of the invention: Insulin derivative and applications for use

2. Claims:
(1) Insulin in which a fatty acid is bonded to an amino group at the B_1 or B_{29} amino acid on the insulin B chain.
(2) Insulin in which a fatty acid is bonded to an amino group at the B_1 and B_{29} amino acids on the insulin B chain.
(3) A drug product in which the active ingredient is a pharmacologically approved quantity of a compound in accordance with Claim 1.
(4) A drug product in which the active ingredient is a pharmacologically approved quantity of a compound in accordance with Claim 2.
(5) A drug product in accordance with Claim 3 or Claim 4 that is an agent for the treatment of diabetes mellitus.

3. Detailed Description of the Invention:
<Industrial field of application>
The present invention concerns a novel insulin derivative, in more detail, an insulin derivative that is useful as an antihyperglycemic in diabetes mellitus.

<Prior art>
Insulin, a peptide secreted by the pancreatic islets of Langerhans and consisting of 51 amino acid groups, is a hormone that regulates the amount of glucose in the blood. When for some reason abnormalities arise in the secretion of insulin from the pancreas, hyperglycemia can develop and lead to a diagnosis of diabetes. In diabetics whose condition is uncontrolled, this hyperglycemic state can lead to a variety of complications, some of which can be fatal. In order to normalize this hyperglycemic condition, it is necessary for the patient to take insulin for amelioration. The insulin taken is in the form of preparations extracted from the bovine or porcine pancreas, preparations of

human insulin obtained from *Escherichia coli* by recombinant genetic engineering, or preparations converted enzymatically from porcine insulin to human insulin.

Human insulin differs from bovine and porcine insulin as shown in Formula I below. Bovine insulin has the amino acids alanine and valine on the A chain at [positions] 8 and 10 (A_8 and A_{10}), and alanine on the B chain at [position] 30 (B_{30}), while porcine insulin has the amino acid alanine on the B chain at [position] 30 and the amino acids threonine and isoleucine on the A chain at [positions] 8 and 10. In human insulin, the amino acids threonine and isoleucine are on the A chain at [positions] 8 and 10, and threonine is the amino acid on the B chain at [position] 30.

When such human, porcine, or bovine insulin injectable is given by subcutaneous or intramuscular injection, the patient's blood glucose can be controlled.

Diabetics must take such insulin injections daily for their entire lives, and this practice is accompanied by considerable physical suffering, including the pain of injection and degenerative changes at the injection site.

In order to reduce the suffering involved with such insulin injection, research is being performed into other [delivery] methods such as oral, perinasal, and rectal administration.

These methods have involved the use of formulation technology to mix the insulin with such substances as absorption promoters and proteolysis inhibitors. Examples include a method of admixture with enzyme inhibitor (Danforth [@@Translator's note: phonetic in source text, spelling assumed] et al.: *Endocrinology* 65, 175, 1978), a method of forming an oil-based emulsification agent (Nanasato et al.: *Acta Diabet. Lat.* 15, 175, 1978), a method using lysosomes (Yoshida: EPA 140,085), and a method whereby insulin granules are coated with azopolymer for release in the colon where digestive enzymes are not secreted (W. Saffran: *Canadian J. Biochem.*, 57, 548, 1979).

Techniques known in the art for the percutaneous sustained delivery of insulin include glycosylated insulin (US Patent No. 4478830, 4478746, 4483792, 4489063, 4489064, and 4536572 Specifications). These various forms of glycosylated insulin [were developed] because crystals precipitated within the conventional insulin injectable preparations, so that those preparations could not tolerate long-term storage.

<Problems the invention is to solve>

The purpose of this invention is to provide insulin derivatives suitable for stable insulin preparations approved as drugs.

<Means of solving the problems>

The inventors of the present invention discovered a novel fatty acid-converted insulin in the form of a fat soluble insulin showing antihyperglycemic action with no loss of insulin activity, and perfected that discovery in the present invention.

The novel insulin derivative of the present invention is represented by Formula I below:

[figure source text page 796, lower left-hand corner]

[key]

A-chain

B-chain

(in which R_1 and R_2 represent identical or different fatty acid groups, X and Y each represent either threonine or alanine, and Z represents isoleucine if X and Y represent threonine and valine if X and Y represent alanine.

Other abbreviations also used in the formula are Phe: phenylalanine, Ile: isoleucine, Val: valine, Glu: glutaminic acid, Gln: glutamine, Cys: cystine, Ser: serine, Leu: leucine, Tyr: tyrosine, Asn: asparagine, His: histidine, Gly: glycine, Ala: alanine, Arg: arginine, Thr: threonine, and Pro: proline.)

The compound of the present invention is useful as an anti-hyperglycemic for diabetes.

The fatty acid-derivatized insulin of the present invention, as shown in Formula I above, has a fatty acid bonded to either or both of the amino acid amino groups at B_1 and B_{29}.

Human, porcine, or bovine insulin can be used as the insulin of the present invention.

Fatty acids that can be bonded under the present invention will preferably have approximately 7 to 21 carbon atoms. These fatty acids include, for example, caprylic acid, pelargonic acid, capric acid, undecylic acid, lauric acid, tridecylic acid myristic acid, pentadecylic acid, palmitic acid, heptadecylic acid, stearic acid, nonadecanoic acid, [illegible] acid, undecylenic acid, oleic acid, elaidic acid, cetoleic acid, erucic acid, brassidic acid, sorbic acid, linoleic acid, and linolenic acid. Palmitic acid is particularly desirable.

The compound of the present invention can be obtained through, for example methods such as those named below.

Procedure 1: Synthesis of activated ester of fatty acid
Procedure 2: Derivatization of the insulin with p-methoxybenzoxy carbonyl azide (pMZ) (formation of pMZ-insulin)
Procedure 3: Bonding of fatty acid activated ester and pMZ-insulin
Procedure 4: Removal of pMZ group
Procedure 5: Separation, purification, and storage

Below, the processes named above are described in more detail.

Regarding the synthesis of the activated ester in Procedure 1, since an unmodified fatty acid is not reactive and will not bond to the insulin in that form, the carboxyl group on the fatty acid is activated to increase reactivity. A specific example would be N-hydroxysuccimide ester.

Regarding the derivatization of the insulin with p-methoxybenzoxy carbonyl azide in Procedure 2, derivatization by a fatty acid of an amino acid on the insulin A chain (Gly:), in particular the amino group at A_1, will reduce the activity of the insulin, so pMZ derivatization is used to protect the amino group.

In Procedure 3, the bonding of the pMZ-insulin obtained in Procedure 2 and the active fatty acid ester obtained in Procedure 1 can be performed satisfactorily at room temperature by mixing within a solvent medium of dimethylformamide.

In Procedure 4, the pMZ protective group that was introduced in Procedure 2 is removed using trifluoroacetic acid.

In Procedure 5, after gel filtration the product is purified by HPLC to yield insulin in which a fatty acid is bonded to an amino group of the amino acid at B_1 or B_{29} on the insulin B chain (insulin bonded with a fatty acid R_1 or R_{29}), or bonded to an amino group of the amino acid at B_1 and B_{29} on the insulin B chain (insulin bonded with a fatty acid R_1 or R_{29})

The resulting insulin derivatives can be subjected to secondary lyophilization to yield a powder.

(Working examples and test examples)

Below, the invention will be explained through working examples. However, this invention is not limited to these working examples.

Reference Example 1 Manufacture of fatty acid activated ester

To 150 ml of ethyl acetate is added 50 mM N-hydroxysuccimide and palmitic acid, after which the mixture is chilled, 50 mM dicyclohexyl carbodiimide is added, and the resulting mixture is stirred for 24 hours. After the reaction is completed, the reaction solution is filtered, and the solvent medium is removed. The residue is then recrystallized from ethanol to yield [illegible, possible misprint for palmitic] acid-N-hydroxysuccimide ester.

Reference example 2 Manufacture of pMZ-derivatized insulin

Bovine insulin at a concentration of 1 mM and p-methoxybenzoxy carbonyl azide at a concentration of 4 mM were dissolved in a mixture of 1N-sodium hydrogencarbonate solution, water, and dimethylformamide (2: 3: 4), and stirred for 3 hours at room temperature. After the reaction was completed, 50% acetic acid was added and the solvent medium was removed. The residue was washed with ether and 1% acetic acid, dissolved in 50% acetic acid, and lyophilized to yield p-methoxycarboxyimide insulin.

Working example

The pMZ-insulin at a concentration of 1 mM was dissolved in dimethylformamide, 50 mM of palmitic acid N-hydroxysuccimide ester was added, and the mixture was stirred for 3 hours at room temperature. After the reaction, the solvent medium was removed, anisole and trifluoroacetic acid were added to the residue, and the mixture was chilled and stirred for 1 hour.

Next, the trifluoroacetic acid was removed, ether was added to the residue, the resulting precipitate was filtered, and the residue was washed with ether.

The resulting residue was dissolved in 1N acetic acid and subjected to gel filtration by passing through a column packed with Sephadex-G25. The insulin fraction was then concentrated.

After the insulin fraction was lyophilized, it was dissolved in a mixture of acetonitrile and 0.3% trifluoroacetic acid (2:3) and passed through an HPLC system to yield Lys-B_{29} palmitoyl insulin (pal-1), Phe-B_1 palmitoyl insulin (pal-2), and Phe-B_1-Lys-B_{29} dipalmitoyl insulin (pal-3).

The results of HPLC are shown in Fig. 1.

The fatty acid binding sites of the insulin derivatives obtained as described above were identified after those derivatives were deaminated, [illegible] degraded, and all peptide bonds had been cleaved to break this substance down into 51 amino acid units. Analysis was performed using an amino acid analyzer.

Values from amino acid were as are shown in Table 1. As the table indicates, the insulin (undegraded product) has free amino groups at 3 sites. If these are deaminated, the amino groups will be eliminated, and cannot be measured by the amino acid analyzer. However, if there is a bond to a fatty acid, deamination does not occur, so when comparing the raw insulin and the deaminated substance there is one more site where the fatty acid bonded, and the binding site can be identified.

[Key to Table 1]

	Insulin			Deaminated pal-insulin		
	Calculated value	Unchanged substance	Deaminated substance	pal-1	pal-2	pal-3

* Diagnostic amino acid

Working example (antihyperglycemic action)

Male Wistar rats were fasted for 24 hours, and were then placed under pentobarbital anesthesia and immobilized on their backs. The test substance was dissolved or suspended in 1N-hydrochloric acid, and animals were injected with this test substance intravenously through the femoral vein or intramuscularly in the femoral muscle. The administered dose was insulin 100 μg/animal. After administration, blood samples were drawn from the carotid artery, and blood glucose levels were measured.

Results are shown in Fig. 2.

As can be seen from the figure, the insulin derivatives Pal-1 and Pal-2 of the present invention produce a remarkable decrease in blood glucose level.

4. A brief explanation of the figures

Fig. 1 is a graph showing the results of HPLC.

Fig. 2 is a graph showing changes in blood glucose following administration.

[Key to figures]
Fig. 1
[vertical axis] Acetonitrile (%)
[dotted line] INS (insulin)
[horizontal axis] Time (min)

Fig. 2
[vertical axis] Rate of reduction in blood glucose (%)

[legend]	[triangle]	:	control (physiological saline)
	[solid circle]	:	pal-2
	[open circle]	:	pal-1
	[box]	:	bovine insulin

[horizontal axis] Time (hr)